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TRAIL induces apoptosis in five of six prostate cancer cell lines. The cell line LNCaP is resistant to TRAIL treatment. Highly constitutive expression of AKT was found in LNCaP cells, and suppression of AKT activity by the PI-3 kinase inhibitors wortmanin and Ly 294002 renders LNCaP cells sensitive to TRAIL. Infection of LNCaP cells with a constitutively active AKT reverses the wortmanin effect. Thus, elevated AKT protects LNCaP cells from TRAIL-induced apoptosis.

Bcl-2 dimerization varies in six different prostate cell lines. Overexpression of bcl-2 in LNCaP cells inhibits cytochrome c release from mitochondria when TRAIL is used in combinaton with wortmanin, suggesting that the mitochondria mediated apoptosis pathway is crucial. The role of mitochondrial superoxide production in TRAIL-induced apoptosis is currently being studied in smooth muscle cells deficient in each superoxide dismutase.

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Table of Contents

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Introduction

TRAIL (TNF (tumor necrosis factor)-related apoptotic inducing ligand) is capable of inducing apoptosis in a variety of human cancers via binding to its receptors DR4 and DR5 (death receptor 4 and 5). It is best known that TRAIL has little or no toxicity to normal cells, which makes it a valuable therapeutic agent in cancer treatment. In this study, we test whether prostate cancer cell lines undergo apoptosis when incubated with TRAIL. We find TRAIL induced apoptosis in five of six prostate cancer cell lines. Moreover, the biochemical mechanism by which prostate cancer cell line LNCaP is resistant to TRAIL-induced apoptosis is examined. Furthermore, the role of mitochondria superoxide production in downstream apoptotic signaling by TRAIL is studied in normal smooth muscle cells system.

Body

In the past year, I received scientific training from the laboratory of Dr. Rodman, division of cardiovascular pulmonary research at the University of Colorado Health Science Center. Through a combination of personal instruction by my mentor and in seminars and journal clubs, I have learned a lot about prostate cancer biology and apoptosis. I also received technical training about generating a transgenic mouse model for apoptosis studies.To date, I have completed task 1 and task 2 proposed in my statement of work. I have established a series of smooth muscle cell lines to work toward finishing up task 3.

My major focus last year was to examine signal transduction pathways in TRAIL induced apoptosis. There are two major apoptotic pathways initiated either by the cell surface receptors (extrinsic pathway) or mitochondria (intrinsic pathway). TRAIL initiates apoptosis through binding to two death receptors DR4 and DR5 on the cell membrane. However, no correlation was found between expressions of DR4, DR5 and the sensitivity of TRAIL in six prostate cancer cell lines. Five prostate cancer cells lines ALVA 31, PC-

3, DU 145, TSU-Pr1 and JCA1 were found sensitive to TRAIL induced apoptosis *in vitro* whereas the prostate cancer cell line LNCaP is resistant.

To study the biochemical mechanisms that cause LNCaP cells to be resistant to TRAIL induced apoptosis, the upstream survival factors PI3 kinase and AKT were tested in prostate cancer cell lines. The TRAIL resistant cell line LNCaP shows a high constitutive AKT activity. Inhibition of PI-3 kinase using wortmanin and Ly 294002 suppressed constitutive AKT activity and sensitized LNCaP cells to TRAIL. Moreover, infection of LNCaP cells with a constitutive active AKT reversed the ability of wortmanin to potentiate TRAIL induced apoptosis in LNCaP cells. Thus, elevated AKT activity becomes the upstream key player that contributes to the resistance of TRAIL-induced apoptosis in LNCaP.

The second element in the TRAIL-induced apoptosis pathway is caspase 8. Caspase 8 is recruited to form the death inducing signaling complex (DISC) along with TRAIL, DR4 and DR5. DISC formation then triggers the autoprocessing and activation of caspase 8. Low expression of caspase 8 was found in TRAIL resistant cell line LNCaP compared to the other five sensitive cell lines. However, caspase 8 cleavage occurred in all six prostate cancer cell lines upon TRAIL treatment. Thus, the block of TRAIL induced apoptosis occurs downstream of caspase 8.

The next element in the TRAIL signal pathway is the substrate of caspase 8, cytosolic proapototic protein p22 BID. BID is cleaved into tBID P15 by activated caspase 8. BID P15 then inserts into the mitochondrial membrane, resulting in the release of cytochrome c. In LNCaP cells treated with TRAIL alone, BID fail to be cleaved, cytochrome c is not released. But BID is cleaved and cytochrome c is released when TRAIL is added in combination with cycloheximide or wortmanin to LNCaP cells. BID cleavage in these cells is consistent with cytochrome c release from mitochondria. Therefore, there is another blocker functions between caspase 8 and BID cleavage.

To more precisely determine the biochemical step at which TRAIL induced apoptosis signaling stops, more study of Bcl-2 family members were done. A major site of activity of the BCL-2 protein is the mitochondrial membrane. Evasion or inactivation of the mitochondrial apoptosis pathway may contribute to resistance of cell sensitivity to TRAIL. BCL-2 inhibits release of mitochondria cytochrome c, thereby promoting apoptosis resistance and cell survival. Overexpression of Bcl-2 in LNCaP cells blocks cytochrome c release but did not affect caspase 8 and BID cleavage and renders the LNCaP resistant to TRAIL plus wortmanin treatment. However, the causes of LNCaP resistance to TRAIL treatment alone are still not clear downstream even though elevated AKT activity is the major upstream factor that mediates TRAIL resistance in LNCaP cells.

There are several bcl-2 family members, which are crucial factors to control the mitochondrial apoptosis pathway. Following TRAIL treatment, proapoptotic molecules such as BAX may dimerize and translocate to mitochondria where anti-apoptotic molecules BCL-2, BCL-Xl always reside. Enforced dimerization of BAX or BAK results in altered mitochondrial membrane potential, production of reactive oxygen species, and release of cytochrome c that activates downstream caspase program. Antiapoptotic factors BCL-2 and BCL-Xl can inhibit the activation of BAX. The ratio of proapoptotic BAX and anti-apoptotic BCL-2 is critical in the TRAIL-induced apoptosis pathway. Bcl-2 dimerization was found to be different in six different cell lines upon TRAIL treatment. Expression of proapoptotic BCL-Xs varies in different cell lines too. BAX expression and its relationship to BCL-2 remain to be tested.

Another bcl-2 family member, proapoptotic factor BAD can be phosphorylated by AKT at Ser 136. Dephosphrylated BAD can form dimers with antiapoptotic bclXl on the mitochondrial membrane, which then signal mitochondrial apoptosis machinery to undergo apoptosis. Phosphorylated BAD fails to form dimers with bclXl and is sequestered in the cytosol by the phophoserine binding protein 14-3-3. BAD phosphorylation status needs to be tested in the six different cell lines to further

demonstrate if BAD is the downstream player that mediates resistance to TRAIL induced apoptosis.

Besides the importance of the above listed members of BCL-2 family in TRAIL-induced apoptosis, mitochondrial dysfunction is important as well. Mitochondrial dysfuction includes a change in the mitochondrial membrane potential, production of reactive oxygen species, opening of permeability transition pores (PTP) and the release of the intermembrane space protein, cytochrome c (Cyt c). One major reactive oxygen species is superoxide anion produced by the electron transport chain in mitochondria. Cells are protected against this metabolically induced oxidative stress by several oxygen radical scavengers, including the superoxide dismutase (SOD). Three forms of SODs are found in mammalian cells, cytosolic Cu-Zn SOD (SOD1), mitochondrial Mn SOD (SOD2) and extracellular Cu-Zn containing SOD (SOD3).

To define the role of mitochondria superoxide production downstream of TRAIL induced apoptosis, three isoforms of SOD knock out mice were generated last year to study TRAIL-induced apoptosis. Mn SOD is the major subject of interest because it is localized within the mitochondria. Mn SOD has been shown to attenuate cytochrome c release and affect downstream caspase 9 activation in the mitochondria dependent apoptosis pathway. Mn SOD can act as a survival factor to protect cells from apoptosis in heptoma cell lines. To test whether MnSOD is the key player downstream of TRAIL- induced apoptosis, primary smooth muscle cell lines were established from heterozygous or homozygous MnSOD mice. As MnSOD-/- mice suffer from severe oxidative damage and the animals die 2 days after the birth, therefore only heterozygous MnSOD+/- smooth muscle cells lines were established. Preliminary study revealed TRAIL induced apoptosis in SOD2 +/- mutant cells. Cyt c release and caspase 9 activation is being tested. MnTBAP, the mimic of SOD2 will be used to test if it rescue TRAIL induced apoptosis in SOD+/- cells.

The emerging evidence suggests that Cu-Zn SOD mutations cause motor neuron cell death. To examine the possibility that TRAIL-induced apoptosis is dependent on Cu-Zn

SOD, smooth muscle cell lines from Cu-ZnSOD+/-, Cu-ZnSOD-/- mice were established. Preliminary research showed lack of Cu-Zn SOD may cause apoptosis in smooth muscle cells, as Cu-Zn SOD-/- smooth muscle cells fail to attach to the tissue culture plate. Further experiments need to test if Cu-Zn SOD enhances TRAIL-induced apoptosis in smooth muscle cells.

Extracellular SOD deficient mice were generated as well but the smooth muscle cell lines have not been established yet. In general, we believe the smooth muscle cell lines generated from the transgenic mice deficient in each isoform SOD will provide us a useful system to test the effect of oxidative damage in TRAIL-induced apoptosis.

Key Research Accomplishments

- **Five of six prostate cancer cell lines were determined to undergo apoptosis when incubated with TRAIL.** Prostate cancer cell lines ALVA31, DU145, PC-3, JCA1 and TSU-Pr-1 were found sensitive to TRAIL-induced apoptosis, whereas the cell line LNCaP is resistant.
- **The mechanism by which the prostate cancer cell line LNCaP is resistant to TRAIL-induced apoptosis is further examined.** High constitutive AKT activity was found in LNCaP cell lines. Inhibition of PI-3 kinase using wortmanin and Ly294002 suppressed AKT activity in LNCaP and sensitized LNCaP to TRAIL. Infection of LNCaP with a constitutive AKT reversed this effect. Thus, elevated AKT activity prevents TRAIL-induced apoptosis in LNCaP cells. Bcl-2 dimerization status varies in all six prostate cancer cell lines. Overexpression of Bcl-2 in LNCaP blocks the release of cytochrome c when LNCaP is incubated with TRAIL in combination with wortmanin.
- **The role of mitochondria superoxide production in TRAIL-induced apoptosis is partly examined.** Transgenic mice deficient in each isoform of SOD (superoxide dismutase), Cu-ZnSOD, MnSOD and EcSOD were generated. Primary smooth muscle cell lines from MnSOD+/- mice, Cu-Zn SOD+/- and Cu-ZnSOD-/- mice were established. Heterozygous deficiency of MnSOD makes smooth muscle cells more sensitive to TRAIL treatment.

Reportable Outcomes

Paper entitled "Elevated Akt activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis" is published in Journal of Biological Chemistry, vol.276, No. 14, pp.10767-10774.

Primary mouse smooth muscle cell lines that carry different SOD mutants MnSOD+/-, Cu-ZnSOD+/- and Cu-ZnSOD-/- were established.

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Elevated Akt Activity Protects the Prostate Cancer Cell Line LNCaP from TRAIL-induced Apoptosis*

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We find that the prostate cancer cell lines ALVA-31, PC-3, and DU 145 are highly sensitive to apoptosis induced by TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand), while the cell lines TSU-Pr1 and JCA-1 are moderately sensitive, and the LNCaP cell line is resistant. LNCaP cells lack active lipid phosphatase PTEN, a negative regulator of the phosphatidylinositol (PI) 3-kinase/Akt pathway, and demonstrate a high constitutive Akt activity. Inhibition of PI 3-kinase using wortmannin and LY-294002 suppressed constitutive Akt activity and sensitized LNCaP cells to TRAIL. Treatment of LNCaP cells with TRAIL alone induced cleavage of the caspase 8 and XIAP proteins. However, processing of BID, mitochondrial release of cytochrome *c*, activation of caspases 7 and 9, and apoptosis did not occur unless TRAIL was combined with either wortmannin, LY-294002, or cycloheximide. Blocking cytochrome *c* release by Bcl-2 overexpression rendered LNCaP cells resistant to TRAIL plus wortmannin treatment but did not affect caspase 8 or BID processing. This indicates that in these cells mitochondria are required for the propagation rather than the initiation of the apoptotic cascade. Infection of LNCaP cells with an adenovirus expressing a constitutively active Akt reversed the ability of wortmannin to potentiate TRAIL-induced BID cleavage. Thus, the PI 3-kinase-dependent blockage of TRAIL-induced apoptosis in LNCaP cells appears to be mediated by Akt through the inhibition of BID cleavage.

TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) (1) also known as Apo-2 ligand (2) is a proapoptotic cytokine that together with three related proteins (tumor necrosis factor-α, CD95L/FasL, and TWEAK/Apo3L) constitutes a family of ligands that transduce death signals through death domain-containing receptors (3–5). TRAIL is a type II transmembrane protein that functions by binding to two closely related receptors, DR4 and DR5 (6). Both TRAIL and its receptors are ubiquitously expressed (7), suggesting the existence of mechanisms that protect normal tissues from TRAIL-induced apoptosis.

TRAIL is capable of inducing apoptosis in a wide variety of cancer cells in culture and in tumor implants in mice, including cancers of the colon, breast, lung, kidney, central nervous system, blood, and skin (1, 6, 8–11). At the same time, unlike tumor necrosis factor-α and Fas ligand, whose use for cancer therapy has been hampered by their severe toxicity (12, 13), TRAIL has no toxic effects when systemically administered in rodents (10) and nonhuman primates (9). Although the majority of normal human cells tested so far appear to be TRAIL-resistant, recent experiments have demonstrated that cultured human liver cells may be sensitive to TRAIL (14), suggesting that additional studies are required to investigate what determines resistance or sensitivity to this agent.

Despite the ubiquitous expression of TRAIL receptors, a significant proportion of cell lines originating from various cancer types demonstrate either partial or complete resistance to the proapoptotic effects of TRAIL. These findings suggest either defects in apoptotic pathways or the presence of inhibitors of TRAIL-induced apoptosis. The latter possibility appears to be more likely, since the resistance of many types of cancer cells to TRAIL can be reversed by treatment with protein synthesis inhibitors (15–19) or chemotherapeutic agents (9, 11). Some normal human cells can also be sensitized to TRAIL by the inhibition of protein synthesis (20). The elucidation of mechanisms that control sensitivity to TRAIL may lead to better understanding of death receptor-mediated signaling and help to develop TRAIL-based approaches to cancer treatment.

Activation of death receptors leads to the formation of the death-inducing signaling complex (DISC)[1] (21), which includes the receptor itself, and caspase 8 (22). The recruitment of caspase 8 to TRAIL receptors DR4 and DR5 is thought to be mediated by the adaptor protein FADD (23–25). The formation of the DISC triggers autoprocessing and activation of caspase 8 (22) that in turn results in the cleavage and activation of the effector caspase 3 or 7 (26, 27), leading to apoptosis. Activated caspase 8 may also cleave a proapoptotic protein BID, whose cleavage product triggers cytochrome *c* release from mitochondria (28, 29). In some but not all cell types, the mitochondrial step may be required to amplify the apoptotic signal and fully activate caspase 8 (30). Since the TRAIL-induced apoptotic signal is a multistep process, inhibition of this cascade may occur at several stages. For example, at the ligand-receptor level, TRAIL signaling could be inhibited by the overexpression of nonfunctional TRAIL receptors DcR1 or DcR2 (31) or by proteins that induce rapid internalization of TRAIL receptors

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[1] The abbreviations used are: DISC, death-inducing signaling complex; PI, phosphatidylinositol; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

(similar to Fas inhibition the adenoviral protein E3) (32). At the DISC, the apoptotic pathway may be inhibited by cFLIP protein that is capable of blocking processing and activation of caspase 8 (33, 34). Downstream of DISC, IAP proteins may specifically inhibit the executor caspases 3 and 7 (35). In those cells that require mitochondria to stimulate apoptosis, the signal may be inhibited by Bcl-2/Bcl-X_L types of proteins that prevent the release of proapoptotic factors from the mitochondria (30).

In the present study, we tested the cytotoxic effects of TRAIL on six human prostate cancer cell lines, demonstrating variable responses, with some cell lines being extremely sensitive and others highly resistant. The highly resistant cell line LNCaP was further investigated to examine mechanisms that protect it from TRAIL-mediated apoptosis. We find that the TRAIL-induced death signal in LNCaP cells is negatively regulated by a high constitutive activity of protein kinase Akt. Furthermore, the antiapoptotic block occurs downstream of caspase 8 activation at the level of BID protein cleavage. This study is the first demonstration that the PI 3-kinase/Akt pathway may interfere with an apoptotic signal by inhibiting processing of BID.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies were obtained from the following sources: anti-phospho-Akt (New England Biolabs, Beverly, MA); anti-cytochrome *c* and anti-BID (Zymed Laboratories Inc.); anti-Akt and anti-XIAP (Transduction Laboratories, Lexington, KY); anti-HA1 tag (Babco, Richmond, CA); anti-caspase 8 (Upstate Biotechnology, Inc., Lake Placid, NY); anti-caspase 7 (PharMingen, San Diego, CA); anti-caspase 9 (Oncogene Research Products, Boston, MA); anti-$FLIP_L$ (Affinity BioReagents, Golden, CO); anti-FLIPγ/δ (Calbiochem).

Cell Culture—Prostate cancer cell lines LNCaP, PC-3, DU 145, TSU-Pr1, JCA-1, and ALVA-31 were passaged in RPMI 1640 with 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin. The sources for these cell lines, their characterization, and use in our laboratories have been described previously (36). LNCaP cells overexpressing Bcl-2 (37) were kindly provided by Dr. R. Buttyan (Columbia Presbyterian Medical Center, New York, NY) and grown in medium supplemented with 400 μg/ml of G418.

Expression of Recombinant TRAIL in Yeast Pichia pastoris—A cDNA encoding for soluble human TRAIL (residues 114–281) was amplified by polymerase chain reaction from the expressed sequence tag clone 117926 (GenBank™ accession number T90422) in frame with the N-terminal hexahistidine tag using oligonucleotides 5′-AGTCATGAATTCCATCACCATCACCATCACGTGAGAGAAAGAGGTCCTCAGAGAGTAG-3′ and 5′-AGTCATGGTACCTTAGCCAACTAAAAAGGCCCCGAAAAA-3′. This cDNA was then cloned into the *EcoRI/KpnI* sites of pPICZαA vector (Invitrogen, Carlsbad, CA) in frame with the cleavable secretion signal from yeast α factor. All manipulations of yeast were performed in general as outlined in the Invitrogen manual. Briefly, the expression vector was linearized and transformed by electroporation into *P. pastoris* strain SMD1168 (38). Transformants were selected on 500 μg/ml of Zeocin, and secretion of TRAIL was tested by Western blotting. For large scale production, yeast were grown for 24 h in 10 liters of complex medium containing glycerol and antifoam 289 (Sigma, St. Louis, MO) and buffered with 100 mM potassium phosphate buffer, pH 6.0, at constant aeration and mixing to A_{600} of 15. To induce TRAIL production, cells were pelleted by centrifugation, resuspended in complex medium containing 0.5% methanol, and grown for 24 h. The supernatant was concentrated using tangential flow Prep/Scale-TFF cartridge (Millipore Corp., Bedford, MA) and recombinant TRAIL purified by nickel-chelate chromatography on a Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen, Valencia, CA). This procedure yielded about 2 mg of pure protein from 1 liter of yeast supernatant.

Cytotoxicity Assays—Cell viability was determined spectrophotometrically using an Aqueous One tetrazolium-based assay (Promega, Madison, WI). Absorbance was measured at 490 nm, and data from duplicate determinations were plotted as percentage of untreated control cells. Quantitative analysis of DNA fragmentation was done using a Cell Death Detection ELISA^{plus} kit (Roche Diagnostics Corp., Indianapolis, IN) by measuring relative amounts of DNA-histone complexes released into the cytoplasm. Data from triplicate determinations were plotted as percentage of control of untreated cells. A TUNEL assay was performed using the FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA).

Measurement of Cytochrome c Release from Mitochondria—Cytosolic extracts from LNCaP cells were prepared by the hypotonic lysis procedure originally described by Bossy-Wetzel *et al.* (39) and modified by Carson *et al.* (40). LNCaP cells grown on 15-cm plates to 50% confluence were placed on ice and then scraped directly into growth medium and centrifuged for 2 min at 200 × *g*. Cell pellets were then washed once with ice-cold phosphate-buffered saline and resuspended in 300 μl of hypotonic lysis buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol) containing protease inhibitors, including Complete Mixture (Roche Molecular Biochemicals, Germany), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. Cells were incubated on ice for 45 min and homogenized by pipetting (10 passes up and down). Supernatants were cleared by 10-min centrifugation at 1000 × *g*, followed by 30 min at 100,000 × *g* and analyzed by Western blotting with the anti-cytochrome *c* antibody.

Construction of Adenoviral Vectors Expressing myr-Akt—The full-length coding sequence of human Akt1 was fused in frame with the myristoylation signal from the human Src protein in the N terminus and HA tag in the C terminus (myr-Akt). Kinase-dead construct was created by mutating lysine 179 for alanine, destroying in that way an ATP-binding site (myr-Akt(K−)). Recombinant adenoviruses were constructed by the method described by Crouzet *et al.* (41). Briefly, cDNAs of interest were subcloned into the expression cassette in plasmid vector pXL2996 under the control of the cytomegalovirus promoter. Each expression cassette was subcloned into the shuttle vector pXL3474. The resulting shuttle plasmids were introduced into *Escherichia coli* JM83 cells by electroporation. After double homologous recombinations, plasmid DNA for recombinant virus was purified by CsCl density gradient centrifugation. This DNA was linearized and transfected into 293 cells. 2–3 weeks after transfections, recombinant adenovirus was harvested from the conditioned medium and amplified in 293 cells.

RESULTS

Effect of Soluble TRAIL on Six Prostate Cancer Cell Lines—Recombinant human TRAIL (residues 114–281) was produced in methylotrophic yeast *P. pastoris* as a fusion protein containing an N-terminal hexahistidine tag and a cleavable secretion signal from yeast α factor. These features allowed quick one-step purification of secreted 20-kDa TRAIL by nickel-chelate chromatography from yeast supernatant yielding ~2 mg of pure protein from each liter of yeast culture medium (Fig. 1*A*). The cytotoxic effects of TRAIL were tested on a panel of six prostate cancer cell lines (Fig. 1*B*). Cell viability assays demonstrated that three of these cell lines, ALVA-31, DU 145, and PC-3 were very sensitive to TRAIL, JCA-1, and TSU-Pr1 revealed moderate sensitivity, whereas LNCaP cells were resistant to as high as 4 μg/ml of TRAIL. Internuclosomal fragmentation (DNA laddering) confirmed that cell death occurred by apoptosis (data not shown).

To investigate the mechanisms controlling the resistance of LNCaP cells to the cytotoxic effect of TRAIL, a series of Western and Northern blot experiments were done to compare the expression of various components of the TRAIL signaling pathway among the six prostate cancer cell lines. However, no correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4 and DR5, decoy receptors for TRAIL DcR1 and DcR2, initiator caspase 8, and apoptosis inhibitory protein cFLIP (data not shown). LNCaP cells contain a deactivating frameshift mutation in the gene encoding the tumor suppresser PTEN (42). This dual specificity phosphatase cleaves D3 phosphate of second messenger lipid phosphatidylinositol (PI) 3,4,5-trisphosphate (43). PI 3,4,5-trisphosphate produced by PI 3-kinase activates protein kinase Akt, and therefore, the lack of negative regulation by PTEN results in the constitutive activation of Akt in LNCaP cells (40). Immunoblot analysis with an antibody that specifically recognizes the phosphorylated/activated form of Akt (Ser^{473}) demonstrates that LNCaP cells possess the highest Akt activity among the six prostate cancer cell lines (Fig. 2*A*). Treating cells with the inhibitor of PI 3-kinase, wortmannin (200 nM), for 6 h

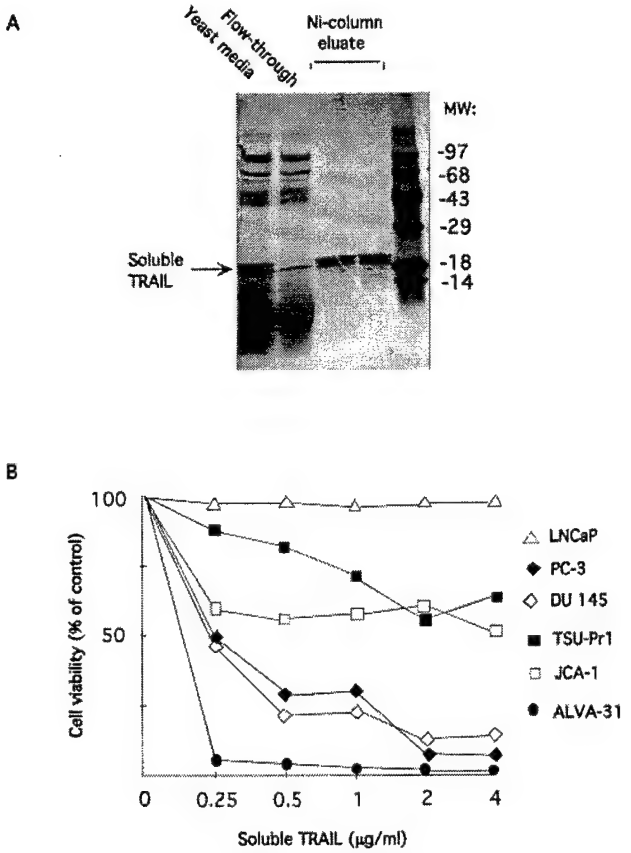

FIG. 1. **Sensitivity of human prostate cancer cell lines to soluble human TRAIL.** *A*, purification of recombinant TRAIL from *P. pastoris* supernatant by nickel-chelate chromatography. *B*, relative viability of six prostate cancer cell lines treated for 24 h with TRAIL, as measured by the tetrazolium conversion assay. Data are expressed as the means for duplicate determinations.

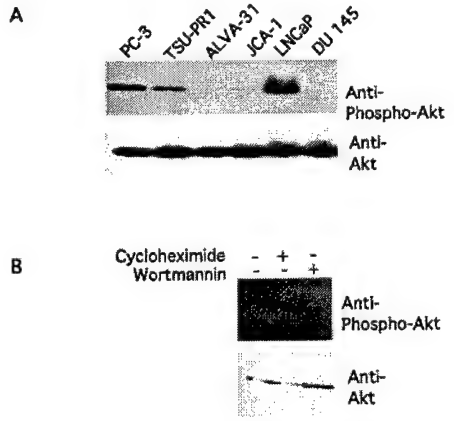

FIG. 2. **Constitutive activity of Akt in prostate cancer cells determined by immunoblot with anti-phospho-Akt antibody (Ser^{473}).** *A*, cell lysates prepared from six prostate cancer cell lines were probed by immunoblotting with anti-phospho-Akt antibody (*top panel*) or anti-Akt antibody (*bottom panel*). *B*, LNCaP cells were treated with wortmannin (200 nM) or cycloheximide (10 μM) for 6 h, and cell lysates were immunoblotted with anti-phospho-Akt antibody (*top panel*) or anti-Akt/PKBα antibody (*bottom panel*).

reverses the high constitutive activity of Akt (Fig. 2*B*).

Inhibition of PI 3-Kinase Activity or Protein Synthesis Renders LNCaP Cells Sensitive to TRAIL—To test whether the high constitutive activity of Akt in LNCaP cells results in their resistance to TRAIL, we first examined how PI 3-kinase inhibitors wortmannin (200 nM) and LY-294002 (20 μM) effect TRAIL cytotoxicity. Wortmannin acts at nanomolar concentrations by covalently modifying PI 3-kinase (44) but is unstable in aqueous solutions (45), making it possible that some PI 3-kinase activity can be restored by *de novo* synthesis in the course of the experiment. LY-294002 does not bind the enzyme covalently and has an IC_{50} value for PI 3-kinase about 500-fold higher than that of wortmannin (46) but is much more stable in culture medium. We have found that both substances significantly enhanced the proapoptotic activity of TRAIL in LNCaP cells as judged by apoptotic morphology (Fig. 3*A*) and DNA fragmentation (Fig. 3*B*), quantitated by measuring the relative amounts of DNA-histone complexes released into cytoplasm. Since wortmannin and LY-294002 inhibit PI 3-kinase by different mechanisms, this result confirms that sensitization of cells to TRAIL occurs through the inhibition of the PI 3-kinase pathway. Inhibition of protein synthesis with cycloheximide also sensitized LNCaP cells to TRAIL (Fig. 3, *A* and *B*). The DNA fragmentation induced by TRAIL in combination with wortmannin, LY-294002, or cycloheximide was greater than that triggered by the potassium ionophore valinomycin (Fig. 3*B*), a potent inducer of apoptosis (47). Thus, the resistance of LNCaP cells to TRAIL results from the blockage of the TRAIL-induced apoptotic signal transduction cascade rather than the defects in apoptotic machinery. These data demonstrate that the inhibition of TRAIL-mediated apoptosis in LNCaP cells requires PI 3-kinase activity and involves some short lived protein component(s).

TRAIL-mediated Cytochrome c Release Is Blocked in LNCaP Cells—Depending on the cell type, apoptotic signaling mediated by CD95/Fas may or may not require the release of proapoptotic factors (cytochrome *c* and apoptosis-inducing factor) from mitochondria. In type II, but not in type I cells, inhibition of mitochondrial apoptogenic activities by overexpression of Bcl-2 protein blocks Fas-mediated apoptosis (30). To examine whether the apoptogenic activity of mitochondria is required for the transduction of the TRAIL-induced death signal in LNCaP cells, the cytotoxic effects of TRAIL alone or in combination with wortmannin were studied in an LNCaP cell line overexpressing Bcl-2 (37). Quantitation of apoptotic nuclei by the TUNEL technique clearly demonstrates that Bcl-2 overexpression impairs the cytotoxic effect of TRAIL (Fig. 4*A*), indicating that mitochondria play an important role in TRAIL-induced apoptosis of LNCaP cells. If the resistance of LNCaP cells to TRAIL results from the high constitutive activity of Akt, this enzyme may block apoptosis either upstream (48, 49) or downstream (50) of mitochondrial cytochrome *c* release. To discriminate between these two possibilities, experiments were done to examine whether TRAIL-induced cytochrome *c* release is inhibited in LNCaP cells. LNCaP cells were incubated for 6 h with TRAIL alone or TRAIL in combination with cycloheximide or wortmannin. Cytosolic extracts were then prepared under conditions that keep mitochondria intact (39), and cytochrome *c* released to the cytosolic fraction was then detected by immunoblotting (Fig. 4*B*). This experiment demonstrated that in LNCaP cells TRAIL alone does not trigger the release of cytochrome *c* from the mitochondria, but it does so in combination with wortmannin and, to a lesser extent, cycloheximide. Thus, TRAIL-induced apoptotic signaling in LNCaP cells is blocked upstream of the mitochondria.

TRAIL-induced Apoptotic Signaling in LNCaP Cells Is Blocked at the Level of BID Cleavage—To understand at what biochemical step the TRAIL-mediated apoptotic cascade is blocked in LNCaP cells, a series of immunoblotting experiments were carried out using antibodies to proteins involved in this cascade. Our results demonstrate that processing of initiator caspase 8 is induced by TRAIL alone as efficiently as when

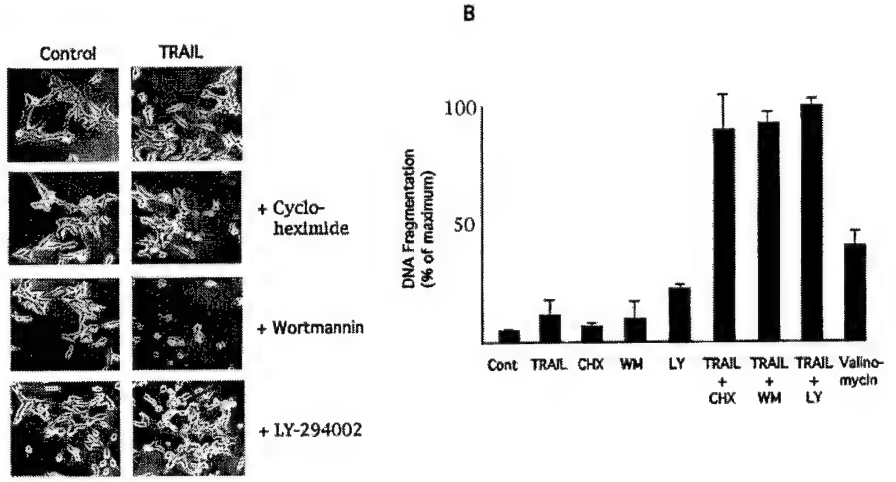

FIG. 3. **Inhibitors of PI 3-kinase or protein synthesis potentiate the cytotoxic activity of TRAIL.** *A*, LNCaP cells were treated for 24 h with 1 μg/ml TRAIL, 200 nM wortmannin, 20 μM LY-294002, or 10 μM cycloheximide alone or in combinations. The cells were visualized by light microscopy. *B*, LNCaP cells were treated for 6 h with 1 μg/ml TRAIL, 200 nM wortmannin (*WM*), 20 μM LY-294002, 10 μM cycloheximide (*CHX*), or 100 μM valinomycin alone or in combinations. DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone complexes released into the cytoplasm using a Cell Death Detection ELISA^{plus} kit.

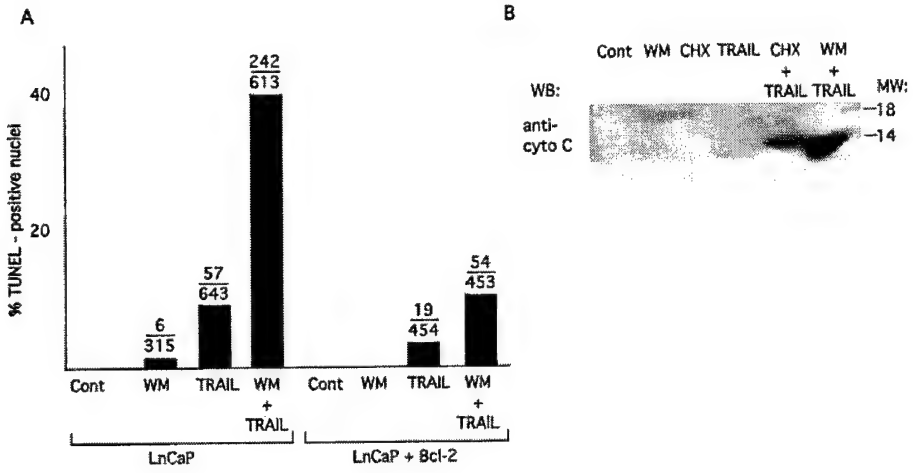

FIG. 4. **The role of mitochondrial cytochrome *c* release for TRAIL-induced apoptosis in LNCaP cells.** *A*, parental LNCaP cells or LNCaP cells overexpressing Bcl-2 were treated as described in the legend to Fig. 3*B*, and apoptotic nuclei were scored by TUNEL staining. Several randomly chosen microscopic fields were visualized, and both normal and TUNEL-positive cells were counted. The numbers of TUNEL-positive *versus* total numbers of counted cells are represented as ratios *above* the *bar graphs*. *B*, LNCaP cells were treated with TRAIL, wortmannin, or cycloheximide as described above. Cells were lysed in hypotonic buffer, and cytochrome *c* in the cytosolic fraction was measured by immunoblotting with cytochrome *c*-specific antibodies.

TRAIL is combined with cycloheximide and wortmannin (Fig. 5*A*). Similarly, these two compounds did not enhance TRAIL-induced cleavage of the apoptosis inhibitory protein XIAP, a substrate for several caspases including caspase 8 (51). These results suggest that the antiapoptotic block in LNCaP occurs downstream of caspase 8 activation. In contrast, proteolytic cleavage of the caspase 8 substrate BID was not detected in TRAIL-treated cells unless TRAIL was administered in combination with cycloheximide or wortmannin. Caspase 8-mediated cleavage of BID generates a proteolytic fragment, tBID, that is capable of inducing mitochondrial cytochrome *c* release and providing a functional link between death receptors and the mitochondria (28, 29). The lack of BID cleavage is thus consistent with the observation that TRAIL alone is not capable of inducing cytochrome *c* release. TRAIL-mediated processing of cytochrome *c*-dependent caspase 9 and effector caspase 7 were also detected only if TRAIL was combined with wortmannin or cycloheximide. The involvement of PI 3-kinase in the blockage of TRAIL-induced BID cleavage was further confirmed by the experiment with another PI 3-kinase inhibitor, LY-294002. Fig. 5*B* demonstrates that treatment of LNCaP cells with LY-294002 in combination with TRAIL results in the decreasing of cellular BID level. Thus, the PI 3-kinase- and protein synthesis-dependent antiapoptotic block in LNCaP cells occurs downstream of caspase 8, at the level of BID cleavage.

Alternatively, it is possible that the lack of BID cleavage may result from an inhibition of mitochondrial function. By analogy with the CD95/Fas system, LNCaP cells may be classified as type II cells, since mitochondrial function appears to be necessary for apoptosis. In type II cells, mitochondrial cytochrome *c* release serves as an amplification loop that potentiates the activation of caspase 8. If a similar mitochondria-dependent amplification loop is involved in TRAIL signaling in LNCaP cells, its disruption may affect caspase 8-mediated BID cleavage. To test whether or not cleavage of BID in LNCaP cells depends on mitochondrial function, the processing of BID in Bcl-2 overexpressor LNCaP cells *versus* parental cells was examined. Immunoblot analysis (Fig. 5*C*) demonstrates that after 6 h of treatment with TRAIL plus wortmannin or TRAIL plus cycloheximide, BID is processed equally well in parental and Bcl-2-overexpressing LNCaP cells. In addition, caspase 8 was processed efficiently in both cell lines as judged by the TRAIL-induced appearance of a cleavage product that corresponds to the 20-kDa active subunit of caspase 8. Thus, apoptogenic activity of mitochondria is not required for TRAIL-induced cleavage of BID and caspase 8.

Our results demonstrate that the blockage of TRAIL-induced apoptosis at the level of BID cleavage can be removed by cycloheximide treatment, suggesting the possibility that this inhibition may be mediated by a short lived protein. It has been hypothesized that inhibition of protein synthesis sensitizes cells to death-inducing ligands by down-regulating antiapoptotic cFLIP proteins (15, 19, 52). To determine whether this is the case for LNCaP cells, cell lysates from a previous experiment (Fig. 5*A*) were immunoblotted with antibodies that recognize different splice variants of cFLIP proteins: $FLIP_L$, FLIPγ, and FLIPδ (53). In contrast to published data, treatment of LNCaP cells for up to 16 h with cycloheximide or wortmannin had no effect on the level of cFLIP proteins (Fig. 5*C*), suggesting that they are unlikely to be involved in the

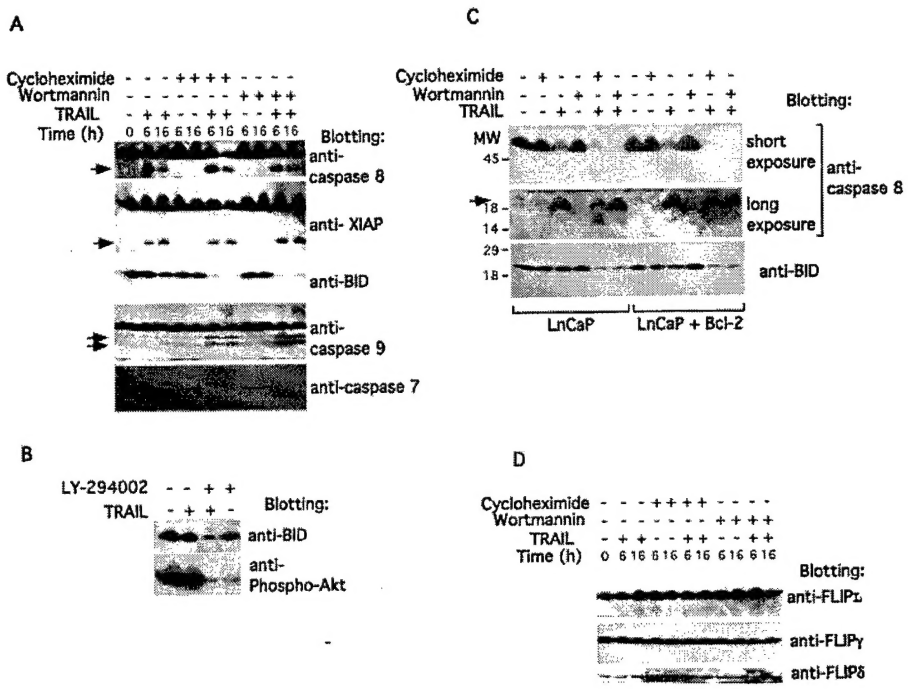

FIG. 5. **Block of TRAIL-mediated apoptotic signal in LNCaP cells occurs at the level of BID cleavage.** *A*, LNCaP cells were treated for 6 or 16 h with 1 μg/ml TRAIL, 200 nM wortmannin (*WM*), or 10 μM cycloheximide (*CHX*) alone or in combinations. Cell lysates were electrophoresed and consecutively immunoblotted with antibodies specific to caspase 8, XIAP, BID, caspase 9, and caspase 7. The *arrows* on the *left* indicate cleavage products. *B*, LNCaP cells were treated for 6 h with 1 μg/ml TRAIL or 20 μM LY-294002 alone or in combinations. Cell lysates were electrophoresed and consecutively immunoblotted with antibodies specific to BID or the phosphorylated form of Akt (Ser^{473}). *C*, parental LNCaP cells and LNCaP cells overexpressing Bcl-2 were treated for 6 h with 1 μg/ml TRAIL and 200 nM wortmannin alone or in combination. Cleavage of caspase 8 and BID was analyzed by immunoblotting with the corresponding antibodies. Blots were processed by ECL, and two different exposures were taken to visualize holocaspase 8 (short exposure) and its 20-kDa proteolytic fragment (long exposure). The *arrow* indicates caspase 8 cleavage product. *D*, cell lysates from the experiment described for *A* were immunoblotted with antibodies that specifically recognize different splice variants of cFLIP protein: $FLIP_L$, FLIPγ, and FLIPδ.

inhibition of TRAIL signaling in LNCaP cells.

Constitutively Active Akt Blocks TRAIL/Wortmannin-induced BID Cleavage—The potentiating effect of wortmannin on TRAIL-induced BID cleavage suggests that Akt may be involved in the inhibition of TRAIL signaling in LNCaP cells. To confirm this hypothesis, a constitutively active Akt, constructed by fusing Akt to the myristoylation signal of Src protein (myr-Akt) was introduced into LNCaP cells by adenovirus-mediated gene transfer. If Akt is the sole target of the wortmannin effect, then this infection would be expected to counteract the ability of wortmannin to sensitize LNCaP cells to TRAIL-induced BID cleavage. As a control, an adenovirus containing kinase-inactive Akt (myr-Akt(K−) was used. LNCaP cells infected with adenoviral constructs 16 h prior to the experiment were treated for an additional 6 h with TRAIL or TRAIL plus wortmannin, and BID cleavage was examined by immunoblotting. Our results demonstrate (Fig. 6*A*) that the infection of LNCaP cells with myr-Akt, but not with the kinase-inactive Akt, inhibits processing of BID induced by TRAIL plus wortmannin treatment. TRAIL-mediated cell death was also inhibited in myr-Akt-infected cells as judged by cell morphology (data not shown). Thus, activated Akt is capable of rescuing LNCaP cells from the apoptogenic action of TRAIL plus wortmannin treatment, supporting the hypothesis that the resistance of LNCaP cells to TRAIL results from high constitutive activity of Akt.

We next tested whether activated Akt can also inhibit cleavage of BID induced by TRAIL plus cycloheximide treatment. However, no rescue was observed even when the adenovirus titer was 16 times higher than that sufficient to inhibit pro-apoptotic effects of TRAIL plus wortmannin treatment (Fig. 6*B*). These results suggest that the protective effects of Akt on BID cleavage may require Akt-induced protein synthesis.

Our results (Figs. 1*B* and 2*A*) indicate the existence of TRAIL-sensitive cell lines that possess an elevated Akt activity, albeit at a much lower level than that found in LNCaP cells. This result raises the question of whether the protective effect of Akt is cell type-specific or it occurs only when the level of Akt activity is above a certain threshold. To examine these possibilities, we overexpressed myristoylated Akt in various TRAIL-sensitive cell lines: DU 145 and ALVA-31 prostate cancer cells, A498 renal cancer cells, and HeLa cervical cancer cells. Of them, only ALVA-31 cells acquired significant resistance to TRAIL upon myr-Akt overexpression (Fig. 6*C*). Thus, the protective effect of Akt appears to be cell type-specific.

DISCUSSION

We have developed a novel approach to obtaining preparative amounts of proapoptotic ligand TRAIL and tested the effects of this reagent on a panel of six prostate cancer cell lines. Soluble TRAIL was produced by a methylotrophic yeast *P. pastoris*, secreted into the medium, and then purified to homogeneity by one-step chromatography on a nickel-chelate column. Cytotoxicity assays demonstrated that three cell lines, ALVA-31, DU 145, and PC-3, were very sensitive to TRAIL, while in comparison JCA-1 and TSU-Pr1 revealed moderate sensitivity, and LNCaP cells were resistant to as high as 4 μg/ml TRAIL. Comparing these results with the data published on Fas ligand-induced apoptosis indicates that prostate cancer cells differ in their responses to these two apoptotic stimuli. Whereas cells believed to be derived from primary prostate cancer tumors (ALVA-31 and JCA-1) were reported to be sensitive to Fas ligand-induced apoptosis, cells originating from distant metastasis (DU 145, PC-3, TSU-Pr1, and LNCaP) appeared to be Fas-resistant despite the expression of Fas antigen on the cell surface (36, 54). In contrast, among the above listed cell lines, only LNCaP cells were resistant to TRAIL-induced apoptosis, indicating that TRAIL has a greater potential as an agent to treat metastatic prostate cancer. These data also suggest that despite the similarity of CD95/Fas and TRAIL receptors, TRAIL and Fas ligand-mediated apoptosis may employ different signal transduction pathways or be negatively regulated by different mechanisms in these prostate cancer cells.

We found that among six prostate cancer cell lines examined, the LNCaP cells, which are the most highly resistant to TRAIL-induced apoptosis, have the highest constitutive activity of the Akt protein kinase. This result is consistent with the lack of the functional tumor suppressor PTEN, a negative regulator of the PI 3-kinase/Akt pathway in these cells (42). Because the Akt

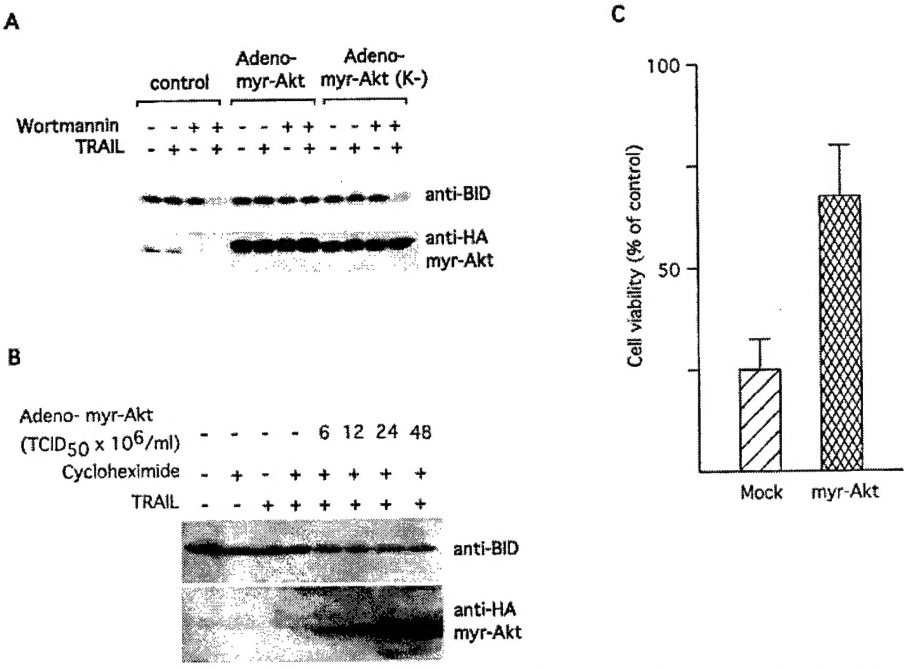

FIG. 6. **Constitutively active Akt inhibits proapoptotic effects of TRAIL.** *A*, LNCaP cells were infected with adenoviral constructs expressing myristoylated Akt (*Adeno-myr-Akt*) or kinase-inactive myristoylated Akt (*Adeno-myr-Akt(K−)*) at a titer of 3×10^6 $TCID_{50}$/ml. Control cells were not infected with adenoviruses. 16 h postinfection, the cells were treated for 6 h with 1 μg/ml TRAIL and 200 nM wortmannin alone or in combination. Cell lysates were consecutively probed with BID-specific antibody and anti-HA1 antibody that recognizes hemagglutinin-tagged myr-Akt. *B*, LNCaP cells were infected where indicated with adenoviral constructs expressing myristoylated Akt (*Adeno-myr-Akt*) at a titer increasing from 6 to 48×10^6 $TCID_{50}$/ml. Control cells were not infected with adenovirus. 16 h after infection, the cells were treated for 6 h with 1 μg/ml of TRAIL and 10 μM cycloheximide alone or in combination. Cell lysates were probed as outlined for *A*. *C*, ALVA-31 cells were transiently cotransfected with an expression plasmid encoding the *E. coli lacZ* gene plus an expression plasmid for myristoylated Akt (*myr-Akt*) (60) or empty expression vector (*Mock*). 24 h after transfection, the cells were incubated with or without 0.1 μg/ml TRAIL and scored for apoptosis 24 h later. Cells positive for β-galactosidase activity were checked for morphological changes characteristic of apoptosis, and the percentage of live cells was quantitated.

protein kinase is known to block apoptosis (55), we tested whether inhibition of this pathway affects the sensitivity of LNCaP cells to TRAIL. We found that treatment with the PI 3-kinase inhibitors wortmannin and LY-294002 or the protein synthesis inhibitor cycloheximide renders them sensitive to TRAIL-induced apoptosis. Thus, the resistance of LNCaP cells to TRAIL results not from defects in apoptotic machinery, but from PI 3-kinase-dependent inhibition of the TRAIL-mediated apoptotic signaling pathway.

It has been reported that apoptosis induced by triggering of CD95/Fas (56, 57) is counteracted by the PI 3-kinase/Akt pathway, but the molecular mechanisms that cause apoptosis resistance remain unclear. To identify which step of the TRAIL-mediated apoptotic pathway is blocked in LNCaP cells, we first tested whether the release of proapoptotic factors from mitochondria is essential for TRAIL-induced death of these cells. The involvement of mitochondria in apoptosis induced by death receptors remains controversial. Scaffidi *et al.* (30) have proposed that two types of cells exist that differ with respect to their requirement for mitochondria during Fas-mediated apoptosis. In type I cells, caspase 8 is activated without involvement of mitochondria to a level sufficient to process the effector caspase 3. In contrast, in type II cells a mitochondria-dependent amplification loop is required to fully activate caspase 8 and transduce an apoptotic signal. This model has recently been questioned by Huang *et al.* (58), who argue that the difference between type I and type II cells is an artifact of using agonistic anti-Fas antibodies to trigger Fas signaling instead of Fas ligand. To clarify the role of mitochondria in TRAIL-induced apoptosis in LNCaP cells, we used Bcl-2-overexpressing LNCaP cells, which were shown to exhibit an impaired cytochrome *c* release in response to various apoptotic stimuli (37). Our results demonstrate that these cells are much more resistant to TRAIL plus wortmannin-induced apoptosis compared with the parental cells. In these experiments, apoptosis was triggered by soluble death receptor ligand and not agonistic antibody, supporting the notion that in some cells mitochondrial function is indeed essential for death receptor-mediated apoptosis.

Using a cell fractionation approach, we have found that TRAIL-induced cytochrome *c* release was blocked in LNCaP cells, but both wortmannin and cycloheximide are capable of overcoming this block. Release of mitochondrial cytochrome *c* by death receptors is triggered by a multistep mechanism. The formation of the DISC results in autoprocessing and activation of the initiator caspase 8 followed by cleavage of the proapoptotic protein BID (28, 29). A proteolytic fragment of BID translocates to the mitochondria as an integral membrane protein and triggers the release of mitochondrial cytochrome *c* (59). Using immunoblot analysis, we found that cleavage of caspase 8 and one of its substrates, the antiapoptotic protein XIAP (51) were induced by TRAIL alone as efficiently as when TRAIL was combined with either wortmannin or cycloheximide. This important result indicates that DISC formation or caspase 8 activation was not blocked in LNCaP cells. In contrast, wortmannin and cycloheximide were required for TRAIL-induced cleavage of BID, the release of cytochrome *c*, and processing of caspases 9 and 7. Thus, the PI 3-kinase-dependent block of TRAIL-induced apoptosis in LNCaP cells occurs at the level of BID cleavage.

The requirement for mitochondrial apoptogenic activity in TRAIL-induced death suggests that LNCaP cells are similar to type II cells. If so, the lack of BID cleavage could, in principle, be explained by the disruption of a mitochondria-dependent

amplification loop, resulting in only partial activation of caspase 8. To see whether this hypothesis could be true, we compared the cleavage of BID and caspase 8 in Bcl-2-overexpressing *versus* parental LNCaP cells and found that these proteins are processed equally well in both cell lines. These results demonstrate that although mitochondrial function is important for TRAIL-induced apoptosis in LNCaP cells, unlike "typical" type II cells mitochondria are required not to amplify caspase 8 activation but to transduce apoptotic signal downstream of the initiator caspase. Therefore, it may be possible to classify LNCaP as type III cells where mitochondria are involved in the propagation rather than the initiation of the apoptotic cascade.

Involvement of PI 3-kinase in the block of apoptosis suggests that Akt could mediate resistance of LNCaP cells to TRAIL. To confirm this hypothesis, we tested whether overexpression of constitutively active Akt could inhibit the proapoptotic effect of TRAIL plus wortmannin treatment. For this purpose, we used a myristoylated derivative of Akt, which exhibits kinase activity independently of PI 3-kinase (60). Both apoptosis (data not shown) and BID cleavage induced by treatment of LNCaP cells with TRAIL plus wortmannin were inhibited by overexpression of myristoylated Akt, indicating that resistance of LNCaP cells to TRAIL is, at least in part, mediated by Akt.

It has been documented that Akt may inhibit a variety of apoptotic stimuli in multiple ways (55). These include direct phosphorylation and modulation of proapoptotic proteins BAD (48) and caspase 9 (50), activation of antiapoptotic NF-κB-mediated transcriptional pathways (61, 62), or phosphorylation of the Forkhead family of transcription factors, preventing them from inducing the transcription of proapoptotic genes (63). Inhibition of BID cleavage has not been previously reported as a mechanism through which PI 3-kinase and Akt block apoptotic signals.

Although it remains unclear how the PI 3-kinase/Akt pathway mediates inhibition of BID cleavage, our data suggest an indirect mechanism. First, inhibition of protein synthesis by cycloheximide affected the same step of TRAIL apoptotic cascade as the inhibition of PI 3-kinase. However, even very high levels of constitutively active Akt did not rescue BID from cleavage when TRAIL was combined with cycloheximide rather than wortmannin. These results suggest that a short lived protein is involved in the PI 3-kinase/Akt-mediated blockage of BID cleavage, and the synthesis of this hypothetical protein may be triggered by Akt. Second, the effect of myristoylated Akt appears to be cell type-specific, since its overexpression did not rescue HeLa, DU-145, or A498 cells from TRAIL-induced apoptosis (data not shown) but did rescue LNCaP and ALVA-31 cells. This could reflect either the difference in apoptotic pathways employed by different cell types or the absence of certain factors required for the protective effect of Akt. In particular, human prostate cancer cell lines have scores of chromosomal deletions and rearrangements (64), so that LNCaP and PC-3 differ in much more than Akt levels.

It has been reported that short term (3–7-h) treatment of human keratinocytes (52), HeLa and Kym-1 cells (19) with cycloheximide significantly reduces the level of cellular cFLIP protein. Since upon overexpression cFLIP is capable of inhibiting Fas-mediated apoptosis (33, 34), it has been suggested that protein synthesis inhibitors sensitize cells to TRAIL by down-regulating cFLIP. To examine this hypothesis, we tested the level of various splice variants of cFLIP ($FLIP_L$, FLIPγ, and FLIPδ) in LNCaP cells and found that neither cycloheximide nor wortmannin treatment affected cFLIP levels after as long as 16 h of treatment. These data are consistent with our observation on renal carcinoma cells (65) and published results on Kaposi's sarcoma cells (17) in which that inhibition of protein synthesis sensitized cells to TRAIL without affecting the expression of cFLIP proteins. Thus, mediators of the PI 3-kinase-dependent blockage of TRAIL-induced BID cleavage and apoptosis in LNCaP cells still await identification and characterization.

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